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Regulation of Transplasmalemma Electron Transport in Oat Mesophyll Cells by Sphingoid Bases and Blue Light[1]

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ABSTRACT

Long-chain sphingoid bases inhibit transplasmalemma electron transport in certain animal cells in part by inhibiting protein phosphorylation. As a first step in determining whether similar regulatory processes exist for cell surface redox activity in plants, peeled leaf segments of *Avena sativa* L. cv Garry were exposed to sphingoid bases and other long chain lipids. Sphingoid bases which are the most active inhibitors of protein kinase C in animal cells inhibit transplasmalemma electron transport by mesophyll cells in the dark as measured by reduction of exogenous ferricyanide. In white light, however, the same compounds markedly stimulate redox activity. The stimulation by sphingoid bases in the light is not eliminated by the inhibitor of photosynthesis, 3-(3,4-dichlorophenyl)-1,1 dimethylurea (DCMU). Redox activity remaining in the presence of DCMU and sphingoid bases can be observed in blue but not red light. A tentative hypothesis considering the involvement of two separate redox systems is presented in an attempt of explain the disparate action of sphingoid bases on electron transport across the plasmalemma.

The transport of electrons across the plasmalemma has been detected in a variety of plant and animal cells (8) and has been implicated in processes triggered by factors such as infection (21), light (10, 22), hormones (5, 15) and iron starvation (3). Also involved in some of these processes are components of the PI[3] pathway and the action of protein kinase C (2), which, in turn, may serve to regulate transmembrane redox activity. For example, after phagocytes are exposed to phagocytosable particles or to certain soluble factors, a mechanism is triggered which activates an NADPH oxidase; the oxidase transfers electrons across the plasmalemma leading to a respiratory burst responsible for the production of superoxide, H_2O_2, and hydroxyl radical (21). This transmembrane redox activity is activated by protein kinase C (21), which is dependent on diacylglycerol, a product of the PI cycle (2). Evidence to support this sequence of events comes from (a) the identification of protein kinase C and PI cycle intermediates in neutrophils and other types of phagocytes (21), (b) the *in vitro* demonstration of a requirement for protein kinase action for NADPH oxidation (21), and (c) the stimulation or inhibition of transmembrane redox by substances known to enhance (analogs of diacylglycerol) or retard (various derivatives of sphingosine) protein kinase C activity (23).

Components of the PI cycle also have been identified in plants (4, 16) and have been shown to be affected by stimuli such as hormones (19) and light (17). Furthermore, protein phosphorylation may mediate certain metabolic events (6, 12). Therefore, we initiated a study to see if the control of transplasmalemma redox activity in plant cells (using exogenous ferricyanide as a probe) is related to that occurring in some animal cells. We will show that the sphingosine derivatives, which are the most potent inhibitors of protein kinase C in neutrophils (23), also inhibit transplasmalemma electron transport in oat mesophyll cells in the dark. Surprisingly, these same substances stimulate redox activity in the light and, in the absence of photosynthesis, the effect is mediated by blue light.

MATERIALS AND METHODS

Culture of *Avena sativa* L. cv Garry plants, preparation of leaf segments, and assay of transplasmalemma redox activity have been described previously (10). Briefly, 7- to 8-d-old light-grown oat leaves were peeled, two 1-cm segments were cut 5 mm from the tip, and the segments were washed in two changes of distilled water. To obtain coleoptiles, oat seeds were germinated in the dark for 4 d with a 30 s exposure to white light on the 3rd d. The coleoptiles were scrubbed with emery (average size 10 μm), washed in distilled water, then isolated and 7 mm segments cut 3 mm below the tip. Root tips were prepared from the apical 5 mm of 2-d-old corn seedlings (*Zea mays* L. cv 'Seneca Chief') as described previously (20).

Fifteen to 20 tissue segments were then placed in 2 mL of medium A (1.0 mM Tris-Mes [pH 6], 1.0 mM KCl, 1.0 mM $CaCl_2$] in the dark for 15 min. After aspiration, the segments were pretreated for 15 to 30 min with 2 mL medium B (medium A now containing sphingoid base or other lipids with or without DCMU). From this point, the experiments were conducted in either of two ways. For method I, medium B was aspirated, and fresh B added now containing ferricyanide to make a final concentration of 0.8 mM. For method II, medium B was aspirated and the segments washed twice with 2 mL aliquots of medium A; after the second wash, medium A was added again, now containing 0.8 mM K_3FeCN_6. Whenever the segments were pretreated with DCMU, it was present throughout the course of the experiment, including any wash-

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[3] Abbreviations: PI, phosphoinositide; DCMU, 3-(3,4-dichlorophenyl)-1,1 dimethylurea; DAG, 1,2-diacylglycerol; PMA, phorbol 12-myristate 13-acetate; RP, redox protein; SB, sphingoid base.

ing steps. In either type of experiment, an aliquot of the ferricyanide-containing solution was removed after 5 to 20 min in the dark or light and the ferrocyanide present measured colorimetrically (1). Manipulations in the dark were carried out under a dim green safe light.

When solutions contained sphingosine derivatives or stearylamine along with ferricyanide (as in method I), a yellow flocculation appeared, and the final incubation medium had to be clarified by filtering through two layers of coarse Fisher brand filter paper prior to assay. Preliminary experiments with either 0.5 mM *erythro*-dihydrosphingosine or 1 mM stearylamine in the presence of 0.8 mM K_3FeCN_6 and known amounts of potassium ferrocyanide showed that, because of the flocculation, the final amounts of ferrocyanide determined after filtering had to be increased by a factor of three. Where this correction is made, it is stated in the appropriate figure or table legend. Flocculation was avoided by using method II.

Stock solutions of the sphingoid bases were prepared fresh daily in 95% ethanol and were warmed before diluting them in the buffered media. After vortexing vigorously, the media were then sonicated in a bath-type sonicator for 2 min. Omitting the sonication step appeared to diminish the effect of the sphingoid base. In both methods I and II, equal amounts of solvent (ethanol for the lipids, methanol for DCMU) were added to the controls. The final solvent concentration in the reaction media varied between 0.5 and 2.0%, and had no effect on redox activity.

Leakage of reductants into the medium was estimated by placing 20 washed leaf segments into 2 mL of medium B containing 0.5 to 1.0 mM of the lipid to be tested. Using a procedure equivalent to method I above, the leaf segments were removed after 15 min, K_3FeCN_6 was added to the medium to a final concentration of 0.8 mM, and the amount of ferrocyanide formed was determined. Alternatively, the segments were placed on medium B for 30 min, washed twice with medium A, and then incubated on medium A for 15 min longer. After removal of the segments, K_3FeCN_6 was added and the extent of reduction measured; this procedure is equivalent to method II described above.

Oxygen exchange in the dark and light was measured at 27°C polarographically using a YSI Clark-type oxygen electrode as previously described (10). Leaf segments were pretreated for 30 min in medium A (control) or medium B (with lipid) as in method I described above, then placed in the electrode chamber containing the same solution with 0.8 mM ferricyanide. Experiments were also performed using method II; the segments were pretreated in medium A or B, then washed in A and transferred to the chamber containing medium A with 0.8 mM ferricyanide. Comparable results were obtained using either experimental procedure.

White light (25 $W \cdot m^{-2}$; 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was provided by two 34-W cool-white fluorescent lamps. Broadband blue and red light was produced with Kodak wratten filters as previously described (10). Exposures were measured with a Li-Cor model Li-185B radiometer.

Chemicals were obtained from Sigma Chemical Company, St. Louis, MO, except *erythro*-dihydrosphingosine, which was from United States Biochemical Corporation, Cleveland, OH.

RESULTS

The protein kinase C and respiratory burst inhibitor, sphingosine, inhibits transmembrane electron transport in mesophyll cells (as measured by rates of ferricyanide reduction) in the dark as little as 5 min after the lipid has been added (Fig. 1). However, the rate of ferricyanide reduction by light-treated tissue is markedly stimulated, but this is only evident 15 min after addition of sphingosine. Another 10 min elapses before ferricyanide reduction reaches its maximum rate.

When mesophyll cells are pretreated in the dark for 30 min in sphingosine and then placed in ferricyanide alone in the light, increased rates of reduction are detected by 5 min, the first time point (Fig. 2). Even though the lipid is no longer present, the stimulated rates in the light are long-lived, as they are retained for at least an additional 20 min (Fig. 2). When the leaf segments are returned to darkness, the rate of ferricyanide reduction drops quickly and approaches the control level within 20 min (Fig. 2). Identical results were obtained for segments preincubated in stearylamine and for those treated simultaneously with sphingosine and ferricyanide (data not shown).

Effects of a variety of sphingoid bases and related compounds on cell surface redox activity are compared in Table I. In these experiments, each compound was tested at 0.5 mM for 30 min and ferricyanide was added to the medium during the last 15 min. Only long-chain, C_{18} alkyl compounds with a free amino group, such as the various sphingosine derivatives and stearylamine, are effective regulators of redox activity. Palmitic acid (C_{16}) which lacks an amino group, octylam-

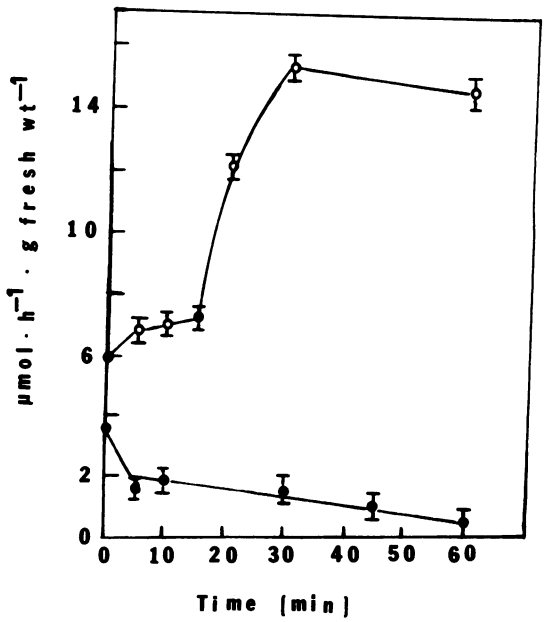

Figure 1. Rates of ferricyanide reduction by oat mesophyll cells after addition of 0.5 mM *erythro*-dihydrosphingosine as a function of time. Leaf segments were placed at 0-time in a solution containing the sphingoid base and 0.8 mM ferricyanide in the dark (●) or in 25 $W \cdot m^{-2}$ (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) white light (○). At the times indicated, the solutions were removed for assay of ferrocyanide and fresh solutions added. Points at 0-time represent rates of ferricyanide reduction by untreated controls. Bars represent ± SD of the mean ($n = 6$).

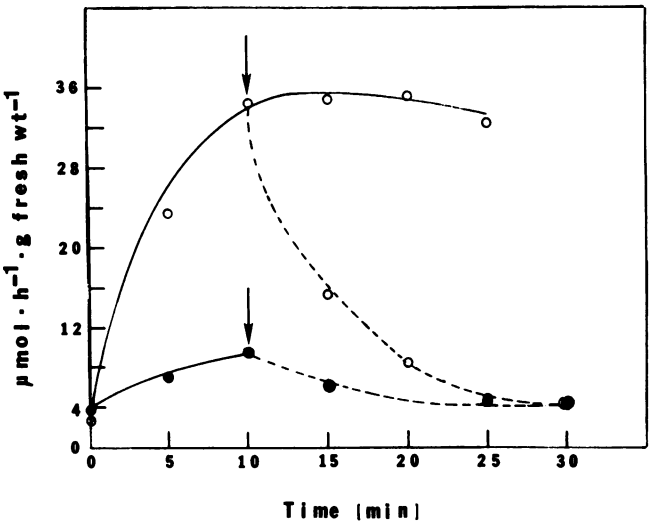

Figure 2. Rates of ferricyanide reduction by oat mesophyll cells after pretreatment and removal of 0.5 mM *erythro*-dihydrosphingosine. Segments were pretreated in the dark with (○) or without (●) the sphingoid base, then transferred at 0-time to identical solutions containing 0.8 mM K_3FeCN_6 without the lipid and placed in 25 $W \cdot m^{-2}$ (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) white light. Arrows followed by dotted lines indicate segments transferred from light back to dark. The SD for each point did not vary more than ±1.2 $\mu mol \cdot h^{-1}$ per g fresh weight from the mean.

Table I. *Effects of Sphingoid Bases and Related Compounds on Transplasmalemma Redox Activity*

Peeled leaf segments were preincubated in the dark for 15 min in solutions containing 0.5 mM of the indicated compound, except for spermine (2.0 mM) and cerulenin (200 $\mu g \cdot ml^{-1}$). After aspiration, the same solutions now containing 0.8 mM K_3FeCN_6 were added and redox activity measured after exposure to dark or light for 15 min. Values are means ± SD for four observations.

Compound	Ferricyanide Reduced	
	Dark	Light (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$)
	$\mu mol \cdot h^{-1} \cdot g$ fresh wt^{-1}	
Control	3.6 ± 0.8	5.8 ± 0.6
D-Sphingosine	0.0 ± 0.1	27.0 ± 0.4
erythro-Dihydrosphingosine	0.6 ± 0.3	24.7 ± 0.8
threo-Dihydrosphingosine	0.2 ± 0.2	18.2 ± 0.3
Phytosphingosine	0.8 ± 0.4	20.9 ± 0.4
Stearylamine	1.2 ± 0.5	11.8 ± 0.8
Palmitic acid	3.3 ± 0.8	7.0 ± 0.3
Ceramide	3.5 ± 0.4	5.9 ± 0.3
Octylamine	4.8 ± 0.0	6.7 ± 0.4
Spermine	3.7 ± 0.8	7.4 ± 0.8
Cerulenin	2.0 ± 0.6	2.3 ± 0.5

ine (a C_8 amine), the C_{10} polyamine, spermine, and ceramide, an *N*-acylated derivative of sphingosine all have little effect.

Phytosphingosine, the naturally occurring sphingoid base in plants is only slightly less effective than the other sphingosines tested. The sterol and fatty acid biosynthesis inhibitor, cerulenin, a C_{12} amide, inhibits redox activity in both dark and light at the concentration used here.

As previously reported (9), less than 10% of the total ferricyanide reduced in the presence of the sphingoid bases is due to leakage of reductants. With octylamine, however, up to 40% of the reduction may be due to leakage. All of the values in Table I have been corrected for any reducing activity lost from the tissue.

Concentration curves for three lipids possessing varying activities as effectors of transmembrane electron transport are presented in Figure 3. The experiments were conducted by pretreating the tissue segments in the lipid for 30 min, then washing and exposing them to ferricyanide alone. In the dark (Fig. 3B), no effect is apparent with any of the compounds under these experimental conditions. In the light (Fig. 3A), it is clear that sphingosine stimulates the rate of ferricyanide reduction and does so at a somewhat lower concentration than that needed for stearylamine. Ceramide is without effect up to 2 mM.

Concentration curves performed with the simultaneous additions of K_3FeCN_6 and lipids give similar results, except now, as also indicated in Table I, sphingoid bases inhibit in the dark. Thus, a relationship seems to exist between the effectiveness of the various lipids as inhibitors of both the oxidative burst and protein kinase C in neutrophils (23) and as inhibitors or stimulators of ferricyanide reduction by mesophyll cells (Table I; Fig. 3). Stearylamine, at 1 mM, was used in many experiments because it was less expensive and more easily obtained than the more active sphingosine derivatives.

No change in the rates of respiration and photosynthesis of leaf cells is seen using the oxygen electrode after pretreating the segments for 30 min in 1 mM stearylamine (data not

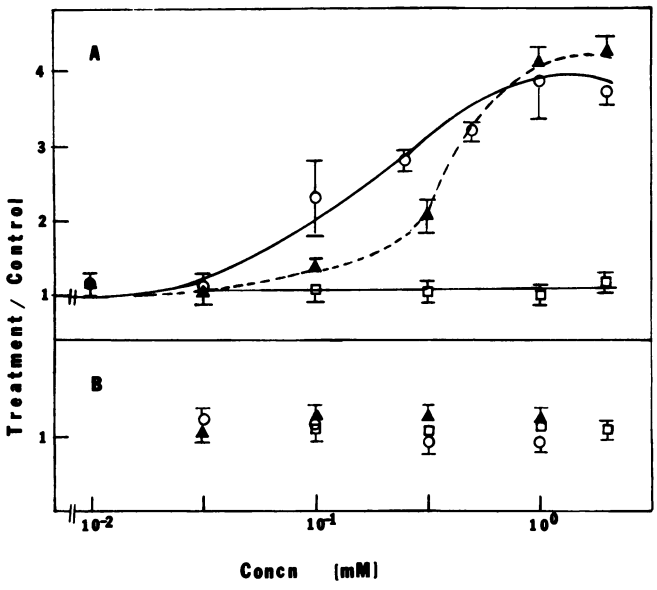

Figure 3. Effects of lipid concentration on rates of ferricyanide reduction by oat mesophyll cells. Leaf segments were pretreated in various concentrations of *erythro*-dihydrosphingosine (○), stearylamine (▲) or ceramide (□) for 30 min in the dark and then incubated in 0.8 mM ferricyanide for 20 min without lipid in 25 $W \cdot m^{-2}$ (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) white light (A) or dark (B). Data were obtained by dividing the rate of ferricyanide reduction for each treatment by the control rate without lipid present. Control rates were 5.4 ± 0.4 $\mu mol \cdot h^{-1}$ per g fresh weight in the light, and 2.8 ± 1.2 $\mu mol \cdot h^{-1}$ per g fresh weight in the dark.

shown). However, the possibility that photosynthesis was indirectly involved in the stimulation of redox activity by light was tested by using red and blue light in the presence and absence of the potent inhibitor of photosynthesis, DCMU. It was first determined that the concentrations of DCMU used (50–75 μM) completely inhibited photosynthesis in leaf segments without affecting respiration.

In the absence of stearylamine and DCMU, the previously reported stimulation of redox activity by white light is observed (10) and, as expected for a process mediated by photosynthesis, white, red, and blue light are almost equally effective ([10]; Table II, column 1). In the presence of stearylamine alone, however (Table II, column 2), only the blue light treatment approaches the rate of ferricyanide reduction in white light, while red is 50% as effective. DCMU, when added alone, eliminates the stimulation by all qualities of light, so that rates in the light are now reduced to the level of the dark controls ([10]; Table II, column 3). But when DCMU and stearylamine are both present (Table II, column 4) higher rates of transmembrane electron transport are seen only in white and blue light. Even when the red light irradiance is increased 3. 5-fold, no stimulation was detected. These results indicate that while photosynthesis may be involved in the sphingoid base-induced stimulation of redox activity in white light, a significant fraction of this stimulation may also be due to a nonphotosynthetic component which is activated by blue light.

A dose-response curve of redox activity in blue light in the presence of stearylamine and DCMU (Fig. 4) shows that half-maximal activity is attained at approximately 17 $\mu mol \cdot m^{-2} \cdot s^{-1}$. This intensity is within the range of some other blue light-induced responses (7, 14).

In order to test for effects of sphingoid bases on transplasmalemma redox in nongreen tissues, maize apical root segments or abraded coleoptile segments were preincubated in 1.0 mM stearylamine; ferricyanide was added after 30 min. Stearylamine inhibits the rate of ferricyanide reduction by root segments from 4.0 ± 0.5 to 1.7 ± 0.7 $\mu mol \cdot h^{-1} \cdot g$ fresh weight^{-1}. Rates by coleoptile segments, however, are stimulated from 50 to 100% over control rates which are 2.0 ± 0.1 $\mu mol \cdot h^{-1} \cdot g$ fresh weight^{-1}. Also, there is little effect of light on root segments; light effects on coleoptiles are currently being investigated.

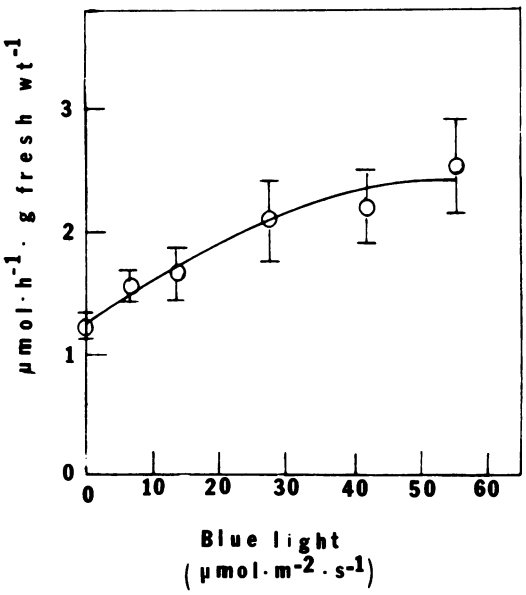

Figure 4. Effect of blue light irradiance on the rate of ferricyanide reduction by oat mesophyll cells. Leaf segments were pretreated in 1 mM stearylamine and 50 μM DCMU for 15 min and then transferred to new solutions containing 1 mM stearylamine, 50 μM DCMU, and 0.8 mM ferricyanide. The amount of reduction was determined after 15 min. Bars represent ± SD of the mean (n = 4).

Table II. *Effects of Stearylamine and DCMU on Transplasmalemma Redox Activity in White, Red, and Blue Light*

Peeled leaf segments were preincubated in the dark for 15 min in the presence and absence of 1.0 mM stearylamine and 75 μM DCMU. After aspiration, the same solutions now containing 0.8 mM K_3FeCN_6 were added and redox activity measured after exposure to the indicated light qualities for 15 min. Quantum flux densities ($\mu mol \cdot m^{-2} \cdot s^{-1}$) were as follows: white, 150; red, 80; blue, 55. Redox rates are corrected for loss of ferrocyanide in the presence of stearylamine (see "Materials and Methods"). Values are means ± SD for four observations.

Treatment	−DCMU		+DCMU	
	−Stearylamine[a]	+Stearylamine	−Stearylamine[a]	+Stearylamine
	$\mu mol \cdot h^{-1} \cdot g$ fresh wt^{-1}			
Dark	2.5 ± 0.3	2.6 ± 1.4	2.5 ± 0.1	3.5 ± 1.1
White	4.4 ± 0.6	29.7 ± 2.7	2.7 ± 0.4	5.9 ± 1.2
Red	4.0 ± 0.5	11.7 ± 3.3	2.6 ± 1.1	3.2 ± 0.9
Blue	4.1 ± 0.6	25.6 ± 4.2	2.6 ± 0.1	6.4 ± 1.2

[a] Data from Dharmawardhane *et al.* (10).

DISCUSSION

The data presented constitute the first evidence that sphingoid bases, which are a normal constituent of the plasmalemma of many cell types, can affect a process in plants—in this case, transplasmalemma electron transport. The most active sphingoid bases inhibit redox activity of mesophyll cells in the dark when present together with ferricyanide (Table I), but stimulate in the light (Table I), even when the cells are only pretreated with the lipid (Fig. 3).

These results appear not to be due directly to toxic effects and/or permeabilization of the membranes (as suggested for neutrophils by Pittet *et al.* [18]) for the following reasons: (a) with the exception of octylamine, less than 10% of the total activity detected is due to leakage of reductant (9) and (b) light has no effect on the small amount of leakage observed (9). (c) The light-induced stimulation of ferricyanide reduction by sphingoid bases is reversible when the cells are returned to darkness (Fig. 2), and (d) the chemicals have no detectable effect on respiration or photosynthesis.

In neutrophils, evidence has been presented that sphingoid bases act by inhibiting protein kinase C, the enzyme requiring DAG and Ca^{2+} for activity and an important regulator of transmembrane electron transport in these cells (23). The possibility that such a mechanism occurs in mesophyll cells can be tentatively suggested, since, regardless of whether redox activity is inhibited (Table I) or stimulated (Table I: Fig. 3),

the relative activities of the sphingoid bases are similar to their effectiveness in inhibiting protein kinase C (23). Furthermore, PMA, an analog of DAG which stimulates protein kinase C and NADPH oxidase activity in neutrophils (21), also stimulates redox activity in mesophyll cells (9).

However, major differences exist between the plant and animal responses to the sphingoid bases. Although they inhibit transmembrane electron transport in root tips and leaf segments in the dark, stimulations are observed using coleoptile segments and leaves in the light; no stimulations have been reported in animal systems. Also, the concentration of sphingoid bases required to elicit a response is noticeably different in the two systems. In neutrophils, the effective range of concentrations is 1 to 50 μM (23), whereas in mesophyll cells it is much higher, 0.1 to 1.0 mM (Fig. 3). These differences in sensitivity to sphingoid bases may reflect biological variations between plant and animal cells and/or differences in the properties of the affected enzyme(s) in the two types of cells. Finally, it should be noted that in neutrophils and epidermoid carcinoma cells, actions of sphingosine other than on protein kinase C have been identified (11, 18). Obviously, further experimentation, including the use of isolated plasmalemma, is needed to determine more precisely the mode(s) of action of the sphingoid bases in plants.

Although it is premature to incorporate the current findings into a comprehensive model, we have devised a working hypothesis in order to focus our efforts for future research (Fig. 5). Any hypothesis must attempt to explain the following observations in mesophyll cells: (a) an inhibition of transplasmalemma redox activity by sphingoid bases in the dark (Table I; Fig. 1), (b) a stimulation by these compounds of redox activity in the light of which only part appears to be dependent on photosynthesis (Table II), (c) a blue light effect evident only in the presence of sphingoid bases (Table II), and (d) stimulations of redox activity by PMA (9).

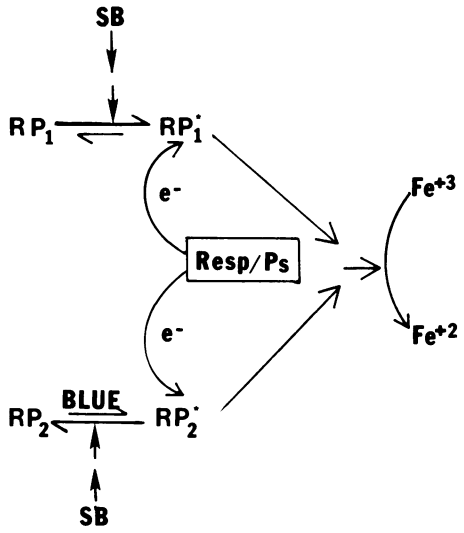

Figure 5. A tentative model to explain effects of certain sphingoid bases on transplasmalemma electron transport in light or dark. RP_1 and RP_2 represent two separate redox protein complexes. Only RP_2 requires blue light for activation (RP_2*). The activation of RP_1 (RP_1*) and the inactivation of RP_2* are inhibited by certain SB, resulting in an inhibition of redox activity in the dark and stimulation in the light in the presence of these compounds. Photosynthesis (Ps) and/or respiration (Resp) provide the electron donor (e^-) for reduction of exogenous ferricyanide (Fe^{+3} to Fe^{+2}). See text for further discussion. Other than the redox protein complexes, which are assumed to be at the plasmalemma, no localization of components is implied by the model.

So as to limit the number and complexity of hypotheses, the following assumptions were made: (a) sphingoid bases and PMA act by modifying protein kinases; (b) two redox protein complexes participate in the response. Evidence for and against the first assumption has been presented above. The possibility that two separate redox systems exist is inferred by data showing that sphingoid bases are effective in the dark after only 5 min, while 15 min must elapse in the light (Fig. 1), and by the fact that when the sphingoid bases are removed, the stimulation of redox activity in the light continues to be observed, while the dark inhibition disappears (Figs. 2 and 3). Multiple redox systems at the plasmalemma have been suggested previously (8).

We propose, therefore, that one redox protein (RP_1) exists which, when activated (RP_1*), mediates transplasmalemma electron transport in the dark; the rate is accelerated by light due to the products of photosynthesis (10). This transport complex is similar to the one proposed by Dharmawardhane *et al.* (10). For optimum activity, RP_1 must be phosphorylated and the protein kinase mediating this reaction is stimulated by PMA and inhibited by SB. This mode of regulation is comparable, at least in part, to that occurring in phagocytes (21).

A second redox protein (RP_2) is also postulated; this protein is activated by blue light (RP_2*) but *inactivated* by a SB-sensitive phosphorylation step—a situation somewhat similar to that reported for rhodopsin (13). Thus, RP_2* would operate in blue light only in the presence of sphingoid bases; activation of RP_1 would be inhibited. In the absence of DCMU, ferricyanide reduction in the presence of sphingoid bases would depend in part on reductant generated by respiration and photosynthesis, but in the presence of DCMU, electrons could still be supplied by respiration.

According to this hypothesis, the differences in the observed redox rates when either RP_1 or RP_2 is functioning, suggests that the two redox proteins differ substantially in their kinetic properties and that RP_2* is the better catalyst of transmembrane electron transport than RP_1*. Variations of this hypothesis could be used to explain the effects of sphingoid bases on roots and coleoptiles.

In conclusion, we have demonstrated for the first time *in vivo*, that transplasmalemma electron transport can be stimulated by blue light. This activity may be the one linked to morphogenic events in plants (24). The nature of the blue light photoreceptor and the effects of sphingoid bases which elicit the response, need to be determined. However, the working hypothesis proposed above allows the design of experiments which may elucidate factors regulating redox activity and which may determine the role of this activity in morphogenic events.

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